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12a. DISTRIBUTION / AVAILABILITY STATEMENT

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13. ABSTRACT *(Maximum 200 words)*

This proposal was to study the structure and function of the yeast plasma membrane H+-ATPase. We proposed to study 1) the kinetics of proton pumping, 2) the transmembrane distribution of the protein and 3) the interaction between the ATPase and an associated protein kinase. Our studies of the proton pumping were unsuccessful due to an inability to reconstitute high activity enzyme into non-leaky vesicles at a high protein to lipid ratio so that proton flux could be measured. The transmembrane distribution project was progressing well until the same project was published by an other group. We have shown a potential link between the level of phosphorylation and the specific activity of the ATPase. We have also shown that the site of kinase mediated phosphorylation is in the C-terminal 5,000 Da of the protein. This domain has previously been shown to be involved in environmentally mediated changes in the ATPase activity. In an effort to produce enzyme with a high protein concentration and high protein/lipid ratio we have begun construction of a Baculovirus expression vector that we will transfect into insect cells. We hope that this system will not suffer from the same low level expression seen in E. coli expression systems.

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Proton Pumping in the Yeast Plasma Membrane H+-ATPase

Final Report

Kirk E. Smith

August 16,1993

U. S. Army Research Office

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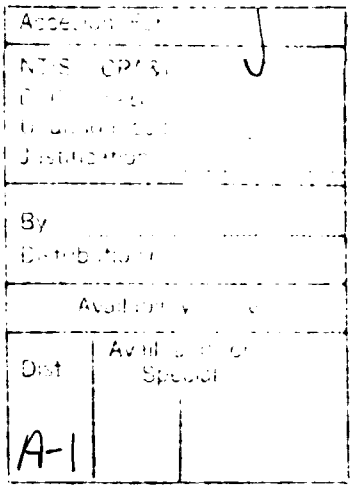

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A. Problem Studied:

This proposal was to study structure/function of the yeast plasma membrane H^+-ATPase. This ATPase is a P-type cation transporter composed of a single protein of 100,000 Da molecular weight. Our original idea was to study three aspects of the structure and function of this enzyme, 1) kinetics of the proton pumping reaction and coupling of proton pumping with ATP hydrolysis, 2) the transmembrane localization of the ATPase protein and 3) the possible regulation of ATPase activity by interaction with an endogenous protein kinase.

B. Results Summary:

1) Kinetics of the Proton Pumping Reaction:

The first step in studying the coupling between proton transport and ATP hydrolysis is developing a reconstituted vesicle system that maintains high enzyme activity and has a high protein to lipid ratio. This is necessary in order to detect the flux of protons across the vesicle membrane. Our success in this aspect was limited. Using a variety of detergents and lipids we were able to purify either high activity enzyme or enzyme with high protein to lipid ratio but not both at the same time.

Our greatest success came from running our crude membrane preparation on sucrose step gradients (10% to 60% in 6 steps) in the absence of detergents. This results in high activity, high protein/lipid ratio material that should be a prime candidate for reconstitution and proton pumping measurements. However, all our attempts to reconstitute this protein into lipid vesicles containing acridine orange resulted in complete loss of ATPase activity. This could be due to inactivation of the enzyme or orientation of the enzyme with the ATP binding site inside the vesicle. At this point the person that was carrying this project left for a better paying job and the project was set aside.

2) Transmembrane Localization of the ATPase Protein:

Our first attempts to describe the structure of the ATPase protein in the membrane involved using complete trypsin proteolysis to identify the membrane protected regions of the protein. The idea here is that those portions of the protein that are inside the vesicle or buried in the bilayer will not be accessible to proteolysis. Since this protein reconstitutes in primarily one orientation (inside out), complete proteolysis should result in a homogeneous population of protected peptides.

We were able to produce and partially purify three low molecular weight peptides that were protected from trypsinolysis. Treating the vesicles containing these peptides with 0.1% triton and re-trypsinizing resulted in the complete digestion of the peptides lending credence to the supposition that they were membrane protected. We were at the point of producing large quantities of peptides to use for HPLC purification of the

individual peptides and sequencing when Scarborough's group (Subrahmanyeswara Roa, U., Hennessey, J. P., Jr. and Scarborough, G.A., 1991, JBC 266:14740-14746) published a very similar study on the ATPase from N. crassa. Since the published results matched ours very closely and also matched the results from limited proteolysis studies on S. cerevesea ATPase we decided to terminate this project.

3) Insect Expression System:

One of the outstanding problems in the study of the S. cer ATPase is getting high enough protein concentrations to do good reconstitutions and structural studies. Previous attempts to surmount this problem by expressing the enzyme in *E. coli* were disappointing (Holzer, K. and Hammes, G., 1989, JBC 264:14389-14395). The bacteria will produce the enzyme but the presence of the enzyme causes the bacteria to stop growing. We decided to try to produce the ATPase in insect cells using the baculovirus expression system hoping that the eucaryotic cells would be more resistant to damage by expression of the ATPase.

We started with a plasmid from Dr. Fink's laboratory that contains the gene for the ATPase (PMA1) along with 3' and 5' flanking regions. We digested this with Hind III and AseI restriction endonucleases to give a fragment of approximately 3000 bp that contains the PMA1 gene and approximately 150 base pairs upstream of the start site. The baculovirus system is supposed to accept up to 200 bp between the promoter and the gene. The AseI site was chosen because it is the nearest to the start site of the gene. The resulting plasmid was purified by extraction from agarose gels, made blunt ended by treatment with Klenow fragment of DNA polymerase and blunt end ligated into the baculovirus transfer vector pVL1393 (Invitrogen Maxbac) previously cut with Sma I and dephosphorylated. This was transfected into competent cells and the cells were tested for transformation by growth in ampicillin containing soft agar. We were at the point of selecting transformants and identifying plasmids when the grant terminated and the person working on this project resigned to go to medical school.

4) Effect of phosphorylation on ATPase:

To study the potential regulatory effect of the endogenous protein kinase on the proton ATPase, it was first necessary to define a means of differentiating between kinase activity and ATPase activity. Both enzymes use MgATP as a substrate so it is difficult to imagine how one activity can be activated without the other. We found that the kinase activity has an apparent K_m of 5μm and is activated by mM $MgCl_2$. The ATPase has a K_m of 5mM and is inactivated when either the free Mg^{2+} or free ATP concentration exceeds 1 mM. Therefore, we were able to kinase treat the ATPase at 10μM ATP, 1mM $MgCl_2$ and be reasonably sure the ATPase was not active at the same time. We could then measure the rate of ATP hydrolysis using either a single time point assay or a coupled assay with lactate dehydrogenase and pyruvate kinase. Comparing the rate of ATP hydrolysis before and after kinase pretreatment gave an indication of the effect of phosphorylation on the ATPase.

Kinase pretreatment results in an increase in ATPase activity that reaches as high as 50% when measured in a steady state enzyme assay. This suggested to us that phosphorylation of the ATPase may be used as an activation signal *in vivo*.

Since it has already been shown that the yeast proton ATPase is regulated by environmental conditions and that this regulation is lost when the C-terminal region of the protein is removed, we wanted to see if phosphorylation also occurs within the C-terminal region. Our first attempts at this involved labeling large quantities of enzyme with ^{32}P, running the labeled protein through gel filtration columns in 1% SDS to remove lipids, precipitation in acetone to remove SDS and trypsin proteolysis. The resultant peptides were then resolved using HPLC. Although we could consistently load several thousand DPM onto the HPLC and get clean consistent peptide maps, we were only once able to identify labeled peptides. In that case the peptides appeared to have resulted from an incomplete digestion. From this we surmised that the site of labeling is in a protease site rich region and that after complete digestion with trypsin, chymotrypsin or elastase the phosphate was attached to either free amino acids, di- or tri-peptides and that these would not resolve in our HPLC system.

We are still working at perfecting either partial digests or other means of identifying the exact location of phosphorylation. In the mean time we have used limited digestion in vesicles to locate the region of the phosphorylation. The ATPase has been previously shown to contain several protease supersensitive sites when incorporated in lipid vesicles. Limited digestion of reconstituted ATPase results in a discreet subset of fragments that are stable enough to isolate and sequence. The location of these protease supersensitive sites have been mapped by sequencing the resulting peptides.

Limited proteolysis of ^{32}P labeled ATPase shows that the first proteolytic cut which removes the C-terminal 5,000 Da of the protein also removes the phosphate. We therefore propose that this phosphorylation may function as a cellular mechanism for regulation of the ATPase activity since it co-localizes to the region of the protein known to be involved in regulation and also seems to have a stimulatory effect on the enzyme. This data has been reported in a manuscript submitted for publication in B.B.A.

C. Publications:

Smith, K. E., Crisp, C. J., Smith, B. C., Nehren, N. J. and Davis, C. (1993) Phosphorylation and Regulation of the Yeast Proton Translocating ATPase. Submitted to BBA

D. Scientific Personnel

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